PB89-233456

Interim Protocol for Testing the Effects of Microbial Pathogens on Lepidoptera

(U.S.) Corvallis Environmental Research Lab., OR

Aug 89

U.S. Department of Commerce
National Technical Information Service
NTIS

PB89-233456

EPA/600/3-89/069
August 1989

Interim Protocol for Testing the Effects of Microbial Pathogens on Lepidopterans

by
J. Miller and B. Lighthart

Oregon State University and
US EPA, Corvallis, Oregon

US Environmental Protection Agency
Environmental Research Laboratory
Corvallis, Oregon 97333

REPRODUCED BY
U.S. DEPARTMENT OF COMMERCE
NATIONAL TECHNICAL INFORMATION SERVICE
SPRINGFIELD, VA. 22161

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA/600/3-89/069	2.
3. RECIPIENT'S ACCESSION NO. PB89-233456/AS	
4. TITLE AND SUBTITLE Interim Protocol for Testing the Effects of Microbial Pathogens on Lepidopterans	5. REPORT DATE August 1989
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) J. Miller and B. Lighthart	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Oregon State University and US EPA, Corvallis, OR	10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS US Environmental Protection Agency Environmental Research Laboratory 200 SW 35th Street Corvallis, OR 97333	13. TYPE OF REPORT AND PERIOD COVERED Published Report 14. SPONSORING AGENCY CODE EPA/600/2
15. SUPPLEMENTARY NOTES 1989. Interim protocol.	

16. ABSTRACT

This protocol outlines methods for laboratory tests on the effects of Microbial Pest Control Agents (MPCA's) on Lepidoptera. It will attempt to cover the mode of action of agents like viruses, bacteria, and fungi in addition to preparations of naturally produced toxins like those of *Bacillus thuringiensis*. Three methods will be used to introduce the agent: contact toxicity, topical application, and ingestion. In keeping with the recommendations of the IOBC, the susceptibility testing will not only evaluate the immediate effects caused by contact, residual, or food-chain mediated mortality but also emphasize assessing the influence on reproduction, development, and survival.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS (This Report) Unclassified	21. NO. OF PAGES 18
	20. SECURITY CLASS (This page) Unclassified	22. PRICE A03

EPA Form 2220-1 (9-73)

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Interim Protocol for Testing the Effects of Microbial Pathogens on Lepidoptera

INTRODUCTION

In 1974, the International Organization for Biological Control (IOBC) established a working group to develop standardized test methods for beneficial entomophagous insects and mites (Franz et al., 1980). The IOBC working group proposed the following approaches to pesticide testing: (1) expose the beneficial insect to a fresh, dry pesticide film applied to an inert surface in order to measure contact toxicity; (2) provide forced ventilation to avoid accumulation of fumes in test cages; (3) use laboratory-reared individuals; (4) apply formulated preparations in the highest concentration registered; and (5) use the performance of the beneficial arthropod rather than the mortality as a measure of pesticide impact. Although all of these recommendations do not directly apply to the testing of microbial agents on Lepidoptera, they do form the basis for establishing specific test procedures. Also, many current pesticide screening techniques reported in the literature can be modified to suit the biology of nontarget species.

One of the many insect-pathogen relationships important to biological control concerns *Bacillus thuringiensis* [BT] and Lepidoptera. Since many species of Lepidoptera are important in the biological control of weeds the use of BT for pest control could create a conflict of interest, particularly in forest, rangeland, and urban situations. For example, BT was applied to over 120,000 ha of wooded urban land in Oregon during a massive program to eradicate the gypsy moth. A three year study demonstrated that population levels of 35 species of nontarget Lepidoptera were effected for a period of two years and species richness was effected for at least three years (Miller, 1988).

This protocol outlines methods for laboratory tests on the effects of Microbial Pest Control Agents [MPCA's] on Lepidoptera. It will attempt to cover the mode of action of agents like viruses, bacteria, and fungi in addition to preparations of naturally produced toxins like those of *Bacillus thuringiensis*. Three methods will be used to introduce the agent: contact toxicity, topical application, and ingestion. In keeping with the recommendations of the IOBC, the susceptibility testing will not only evaluate the immediate effects caused by contact, residual, or food-chain mediated mortality but also emphasize assessing the influence on reproduction, development, and survival.

Since adult Lepidoptera either do not feed or are nectar feeders, contact infection is relatively minimal in comparison to the potential for exposure of larvae to MPCA's. However, the potential for infection of adults could occur with MPCA ingestion during feeding on plant nectar or on additives in formulated MPCA

---

*Disclaimer: This interim protocol was developed on the basis of current kowledge of testing the toxicity of chemical pesticides to insects. Modifications may be required as experience in testing microbial pesticides is acquired.

mixtures, such as, molasses. Also, adult Lepidoptera may contact an MPCA while walking or resting on various substrates. Also, eggs and pupae may be exposed to MPCA's through contact either on a substrate or topically. The primary stage affected by contact and oral (injestion) infection are the herbivorous larvae. Larvae typically possess three pair of short, segmented thoracic legs and five (sometimes two, three, or seven) pairs of unsegmented abdominal prolegs. The morphology of the larvae (caterpillars) thereby creates a condition of extensive contact with a treated substrate. Also, larvae will feed on plant foliage resulting in the ingestion of a MPCA present on the plant.

Sublethal pathogenic effects would consist of: slower and or less movement, decreased feeding rates, lighter body weights, slower development, incomplete pupation, unsuccessful emergence of adults, reduced longevity, reduced success at mating, and lowered fecundity.

GENERAL BIOLOGY OF THE LEPIDOPTERA

The Lepidoptera exhibit a holometabolous life cycle with 4 life stages: egg, larva, pupa, and adult. Adults mate, disperse, and females lay eggs. Larvae feed while developing into pupae through a series of instars. Pupae develop into adults. The location and duration of each of these stages affects potential exposure to MPCA's and varies widely among the species. The eggs of certain species may hatch in 4 days while in other species eggs may hatch in 8 months, perhaps involving a state of diapause. Development of pupae may exhibit the same types of patterns as just described for the eggs. Larvae typically develop through 5-7 instars with the last two instars consuming 75-85% of the total nutritional requirements necessary for adulthood. All of the life stages may be very well concealed inside various substrates where a MPCA may not penetrate or directly exposed to an open environment where MPCA's would be present.

Criteria for Selection of the Test species. There are about 112,000 species of Lepidoptera in the World and 11,000 in North America (Borror and White, 1970). In general species of Lepidoptera are herbivorous. The following criteria are used to select the proper test animal.

Habitat. The test animal should frequent the same crop type (orchard, field, vegetable, and ornamental) for which the agent is (to be) registered.

Availability. The animal must either be caught or bought in sufficient numbers to start a laboratory culture. A culture is suggested because it produces animals of known age, sex, and physiological conditioning.

Requirements for Rearing. The test organism, a species of moth or butterfly, must have a life cycle suitable to laboratory rearing. It must be reared through all life stages on an artificial diet or on a host plant that can be easily cultured in a lab or greenhouse clean of possible contaminants. The test organism must be free of disease.

Suggested Test Animal. The variegated cutworm, *Peridroma suacia*, is a common species in North America and is easily cultured. Although this particular species is considered a pest, it serves as a model for the protocol to test nontarget species of Lepidoptera. Adults mate readily in 3-liter jars and females will deposit eggs onto wax paper and paper toweling. Eggs will hatch in 4-5 days at 22°C. Larvae develop through 6-7 instars in 25-38 days at 22°C while feeding on an artificial diet based on agar and pinto beans (Shorey and Hale 1965). Pupae

require 14-20 days at 22°C before eclosion of adults (Shields 1983). Adults will live for 10-20 days at 22°C if provided a 5% solution of sugar (honey may be used) in distilled water.

TEST PROCEDURES

The proposed tests refer specifically to the variegated cutworm and are designed to standardize various environmental conditions so that MPCA effects may be ascertained.

Test Conditions

During testing, both larvae and adults should be kept in a growth chamber (photoperiod of 16L:8D, 50-80% RH, 22-24°C). Temperature and humidity should be adjusted within these ranges for optimal effectiveness of the MPCA. Adults must be provided food resembling a nectar source, such as, 5% honey-water soaked onto cotton. Adults (two pairs of females-males) can be put into vented (one-liter to four-liter) containers. Jars or plastic containers with screened tops are most suitable. Tight, closed containers should not be used becuase of fumigation effects and problems with excessive moisture. Also, the adults should be provided a means for obtaining fresh air.

Regular disinfection of containers is essential to preventing unwanted micro-organisms from being present. Containers and cages can be cleaned with disinfectant soaps or 95% ethanol followed by two rinsings with distilled water. Also, equipment, instruments, and supplies must be sterilized (in an autoclave) and aseptic techniques used throughout all procedures.

Stock colonies must be separated physically (separate laboratory or building) from test area, where contact with MPCA's could occur.

As a general rule, if larval or adult control mortality is highly variable among replicates and is greater than 20%, or successful pupation is less than 75%, then the colony may be diseased or otherwise unfit thereby invalidating test results.

Number of Individuals in Treatment Groups

The test should include at least 100 individuals per treatment rate with the same number in the control group. The test organisms should be obtained from at least five different mated females. In general, 20 to 25 larvae should be tested per replicate and the tests repeated 4-5 times.

Preparation and Quantification of Test Materials

The MPCA should be prepared using the manufacturer's directions. Initially, rates tested should include 1/10 the field rate, the proposed field rate, and 10 times the field rate. If significant mortality is observed at the lowest rate, then a dose response should be conducted using at least 5 treatment rates and a water control. The highest dose should correspond to the treatment of 1/10 field rate from the original test. Use distilled water throughout all preparations. The water control treatment must include heat-inactivated MPCA. The MPCA may be inactivated by heating a water suspension of the test material to 95°C for 10 minutes. A test to verify inactivation of the MPCA should be conducted using culturing techniques appropriate for the MPCA. A lack of success in culturing the heat treated MPCA will indicate successful heat-inactivation.

When performing any of the microbial procedures used in this protocol, it is recommended that the Class II containment procedures be followed as specified in Biosafety in Microbiological and Biomedical Laboratories (Birchardson and Barkley, 1984).

ASSESSING RESPONSE LEVELS

The response of the test organisms may be classified into three levels: 1) acute lethal effects, 2) sublethal effects, and 3) no effect. All response levels must be closely monitored and documented.

Response Level I - Acute lethal effects

Mortality should be recorded every 24 hours after exposure until death for eggs, larvae, pupae, and adults. Death is indicated if the larva or adult does not respond to probing. Egg and pupal death would be indicated by a lack of emergence of a larva from the egg and a moth from the pupa after a week following the last individuals in the control treatment. It will be necesary to verify mortality by dissecting eggs and pupae presumed to be dead. If high mortality occurs at the prescribed doses and time intervals, a more extensive serial dilution (up to 5 or 6 dose levels) is required to determine the lethal concentration (LC_{50}).

Statistical analysis for Response Level I. The 0.1x, 1.0x, and 10x field rates should be compared using an F-test. The dose-mortality data should be evaluated with a probit analysis (Finney, 1977). The statistical output includes the number of animals tested at each dose, the lethal concentration for 50% mortality with 95% confidence intervals, and the standard error of the slope.

Response Level II and III - Sublethal effects / No effects.

If no mortality occurs after 72-120 hours, then sublethal effects should be monitored as described below. A sublethal dose level needs to be established in order to conduct these tests.

EGGS- After exposure to the sublethal dose and heat-inactivated MPCA control, eggs are maintained in units of 10 each in rearing chambers. Observations are to be made on percent hatch of eggs, time to hatch, and survival of the young caterpillar through the first instar (fed a noncontaminated artificial diet).

LARVAE- After exposure to the sublethal dose and heat-inactivated MPCA control, newly-hatched larvae are maintained singly in rearing chambers and fed a premeasured quantity of diet. The diet consumption (daily and total), as well as duration in each larval stage (days), is recorded until pupation. Percent pupation is also noted. Pupal weights should be assessed. If adults are obtained they should be monitored in the manner as described below.

PUPAE- After exposure to the sublethal dose and a heat-inactivated MPCA control, the pupae are maintained singly in rearing chambers. Observations are to be made on percent emergence of adults from the pupae, time to adult emergence, and survival-etc. of adults as mentioned below.

ADULTS- After exposure to the sublethal dose treatments and a heat-inactivated MPCA control, mated females are maintained singly in rearing chambers and fed 5% honey-water. The following should be recorded: percent survival on a daily basis, number of eggs deposited per female, egg viability as determined by percent hatch, and adult longevity.

Statistical Analysis For Response Level II. Survival (percent egg hatch, pupation, adults) may be tested by a chi-square test or an F-test to determine treatment differences from the control tests. Differences in developmental times by treatment should be tested for with an F-test.

TESTING METHODS

Three MPCA exposure methods (application techniques) need to be tested: contact, topical, and ingestion. Each life stage, egg, larva, pupa, and adult, is to be tested.

TEST I: Contact Toxicity.

Eggs, larvae, and adults are tested. The procedure for effectively evaluating this technique is to evenly coat the inner surface of a test chamber with a known quantity of MPCA in suspension so that the test animal cannot locate an untreated area for refuge.

EGG- Monitor the responses of the eggs after they have been deposited on freshly treated/untreated surfaces. The inside surface of a 1 liter container is treated with the MPCA; a single mated female moth per container is allowed to deposit eggs for a 12 hour period (n=10 moths); to maintain a standard sample size remove all but 10 eggs per container and monitor the acute and sublethal responses as described above.

LARVAE- Young larvae of each instar: I, II, III, IV, and V (to standardize age and physiological effects all larvae used should be within 8 hr of egg hatch or molt from the previous instar) should be placed singly in treated chambers. After 24 hours of exposure (do not provide diet during exposure period) remove the larvae and rear them individually. A pathogen and antibiotic-free diet must be provided during the remaining larval development.

ADULTS- Place moths (recently emerged from the pupa, <8 hours old) in chambers singly or in groups of five if large test chambers or petri dishes are used. Equal numbers of both sexes are tested. After 24 hours of exposure to the MPCA the moths are removed from the exposure-container and placed in clean rearing-containers.

TEST II: Topical Application.

Eggs, larvae, pupae, and adults are tested. The procedure for effectively evaluating this technique is to place a precise amount of the MPCA directly onto the body of the test organism. A common procedure is to use a calibrated syringe or microapplicator (Coats et al., 1979). The agent is applied in concentrations above (10x), below (0.1x), and at (1.0x) the recommended field rates. The control is a dose of the heat-inactivated MPCA at each treatment rate. If adjuvants (surfactants, stickers) are required to promote MPCA action under field conditions, it is important to incorporate them as a treatment. Thus, an additional control treatment consisting of the adjuvant and heat-inactivated agent is needed. When performing any of the microbial procedures it is recommended that the pathogen be suspended in water.

Although a microapplicator is the common instrument for dispensing non-MPCA doses, no specific body part is standard for receiving the application. This is probably a trivial point when applying a microliter of technical grade pesticide in acetone, but may take on more meaning when dealing with larger quantities of biological MPCA-agents suspended in water.

EGGS- Monitor the responses of the eggs after they have been deposited on an untreated surface. Apply the calibrated dose to the top (micropylar area) of each egg.

LARVAE- Apply the MPCA to the dorsal thoracic segments of the larva. The thorax provides a large surface for the carrier to evaporate and keeps the agent away from the mouth parts, thus avoiding ingestion. To assure the proper exposure rate, the test individuals must not be allowed to move and rub off the

agent before drying. Larvae should be chilled in a refrigerator (ca.9-11°C) for 5 minutes to reduce their movement during the application. The effects of chilling may need to be tested as well (Lighthart et al. 1988)

PUPAE- Apply the MPCA to the dorsum of the thorax. Then place pupae singly in clean rearing units.

ADULTS- Remove the hair or scales from the ventral surface of the first three abdominal segments. This may be performed by using a scalpel or stiff haired brush. Then apply the desired concentrations of the MPCA to the dehaired area. The adults should be anesthetized with CO_2 just prior to the dehairing and application activities. A control treatment involving the CO_2, dehairing, and MPCA carrier should be conducted.

TEST III: Ingestion.

Only larvae and adults are tested. The procedure for effectively evaluating this technique is to present the MPCA in combination with a food source at a rate that will reflect a measure of toxicity after ingestion.

MPCA coverage or incorporation into the diet, diet nutritional quality, and consumption rates are variables that must be monitored and standardized as much as possible. Larvae and adults of both sexes are fed a contaminated food source until the larvae pupate or the larvae/adults die.

LARVAE- Depending on the type of MPCA being evaluated,one of two tests may be most appropriate. These tests differ in the method of exposing the larva to the MPCA: 1) clipped foliage, 2) incorporated into an artificial diet.

The quantification of the dose actually injested by the larva is very important in these tests. Measurements must document the quantity of food consumed so the actual amount of MPCA injested can be determined. Also, periodic assessment on the viability of the diet incorporated MPCA should be conducted following culturing techniques appropriate to the type of MPCA being used.

Clipped foliage. In this test the food source is in the form of live plant material. Twigs or individual leaves of a suitable species are provided to the larvae from the time of egg hatch. The test is performed as described below and then larvae are again reared on MPCA free foliage. Tests are to be conducted with foliage from only one plant species at a time. Tests comparing the influence of foliage from different plant species on MPCA effects would provide valuable information on the need to mitigate the pathogen.

Foliage from a suitable host plant is clipped with the stem immediately tightly packed into a tube of water. The foliage then is dipped into a solution of the MPCA previously mixed to the appropriate concentration. Allow the foliage to air dry and then place a single larva on the foliage. The amount of MPCA injested can be calculated from data on quantity of MPCA per leaf area and the leaf area consumed by the larva. The amont of MPCA on the leaf can be determined by 1) staining and counting the density of organisms on the leaf surface, or 2) washing the treated leaves and plating the organisms on an agar substrate suitable for growth of the particular MPCA being tested. The amount of leaf area consumed may be determined by comparing the leaf area initially provided the larva and the leaf area remaining after 24 hours. A leaf not exposed to the larva must be measured to correct for slight degrees of leaf shrinkage over the 24 hour period of the test under identical conditions. A Li-Cor area meter or digital graphics board is best for the area measurements.

Artificial diet. The diet needs to be made and cooled prior to incorporation of the MPCA. When cooled (to about 55°C) but not hardened the appropriate amount of MPCA is to be mixed into the diet and then left to solidify. Tests must be conducted to verify viability of the MPCA after incorporation into the diet at

the prescribed temperature. Once prepared the diet can be stored at a cool temperature (ca. 10-15°C) and cut into small cubes for eventual feeding. If the storage life of the MPCA does not allow for refrigeration then diet must be prepared daily. The amount of MPCA injested can be calculated from data on the quantity of MPCA in the diet and the amount of diet consumed. Use dry weight equivalents in these calculations. When conducting a test involving artificial diet remember to avoid antibiotics, heat (cooking) during or after MPCA incorporation, and use a standardized nitrogen level. The nitrogen content in the foliage of most plant species ranges between 1% and 4% N by dry weight. Inconsistent levels of nitrogen among the tests could influence MPCA effects. Diet cubes must be changed daily.

ADULTS- Incorporate the appropriate concentration of MPCA into the honey + water solution used to feed adults. Supply the treatment for the entire period of adult life. The quantification of the dose actually injested by the adult is very important in this test. The amount of solution consumed by the adult must be measured (with corrections for evaporation).

REPORTING RESULTS

Test data should be recorded as described and include:

1. Name of the test, sponsor, test laboratory, study director, principal investigator, and dates of testing.

2. A detailed description of the test MPCA, including the formulation concentration, and lot number or batch number. Include the type of dilutions carried out.

3. Detailed information about the source of test organisms used, and history of indigenous disease, if known. Age of test subjects, test conditions (temperature, lighting level and duration, and relative humidity) should be included, as well as the exact method of dosing.

4. A description of the test substrates, dimensions, number of test organisms per cage, and number of replicates per dilution of MPCA.

5. The percentage of test subjects dead at each dose after each observation period.

6. The mean duration in days of the larval period, the percentage successful emergence of adults, and the number of eggs and the percent egg hatch for long-term tests of larvae.

7. The statistical test used if a dose response is conducted. Minimal data reported would include the LC_{50} and confidence interval, the slope, the intercept, and number of test subjects.

8. A report of any deviation from this test protocol, as well as anything unusual about the test such as temperature fluctuations, disease problems in the test subjects, etc.

REFERENCES

Birchardson, J.H. and W.E. Barkley. (eds.) 1984. Biosafety in Microbiological and Biomedical Laboratories. U.S. Dept. Health and Human Services, Center for Disease Control, Atlanta, GA.

Borror, D.J. and R.E. White. 1970. A Field Guide to the Insects of America North of Mexico. Houghton Mifflin Co. 404p.

Coats, S.A., J.R. Coats, and C.R. Ellis. 1979. Selected toxicity of three synthetic pyrethroids to eight coccinellids, a eulophid parasitoid, and two pest chrysomelids. Envir. Entomol. 8:720-722.

Finney, D.J. 1977. Probit Analysis. Cambridge University Pr., Cambridge, England. 333pp.

Franz, J.M., H. Bogenschutz, S.A Hassan, P. Huang, E. Nation, H. Suter, and G. Viggiani. 1980. Results of a joint pesticide test program by the working group: Pesticides and beneficial arthropods. Entomophaga 25:231-236.

Lighthart, B., D. Sewall, and D.R. Thomas. 1988. Effect of several stress factors on the susceptibility of the predatory mite, *Metaseilus occidentalis*, to the weak bacterial pathogen, *Serratia marescens*. J. Invert. Pathol. 52: 3-42.

Miller, J.C. 1988. Impact of BT applications for the control of the gypsy moth in Oregon: Affects on nontarget Lepidoptera. A Final Report to the Oregon Dept. Agric.

Shields, E.J. 1983. Development rate of variegated cutworm. Ann. Entomol. Soc. Am. 76: 171-172.

Shorey, H.H. and R.L. Hale. 1965. Mass rearing of the larvae of nine noctuid species on a simple artificial medium. J. Econ. Entomol. 58: 522-524.